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(54) ENVIRONMENTAL STRESS RESISTANT PLANT AND ITS PRODUCTION

(57)Abstract:

PURPOSE: To produce a plant reinforced in resistances to environmental stresses such as a high osmotic pressure, a low temperature, a high temperature and a dryness by introducing a choline dehydrogenase gene, etc., related to the synthesis of glycine betaine, into a plant.

CONSTITUTION: This method for producing a plant reinforced in resistances to objective environmental stresses, comprises introducing a choline dehydrogenase gene and a betaine aldehyde dehydrogenase gene obtained from bacteria and related to the synthesis of glycine betaine into the plant of trees, grasses, etc., such as a tobacco and a rice.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the vegetation by which the resistance over the stress by various environmental stress, for example, a hyperosmolarity, low temperature, the elevated temperature, desiccation, etc. was reinforced, and its creation approach.
[0002]
[Description of the Prior Art] The vegetation which exists in a nature is exposed to various environmental stress, such as salt stress, desiccation stress, elevated-temperature stress, and low-temperature stress, and in the process of evolution, vegetation acquired the device in which it could respond to the environmental stress of the area where vegetation grows wild, and has evolved. On the other hand, improvement of a species is performed by artificial means, such as mating, with agricultural development, and the form which became strong a little at environmental stress is also made. However, still sufficient correspondence has been performed neither with a desert nor a salt damage background.
[0003] It is known that the glycine betaine which is one sort of the adaptation solute of a low-molecular organic compound will participate in the osmoregulation in a plant cell. In the biosynthesis of glycine betaine, a choline is converted into the basis of an intervention of choline dehydrogenase by the betaine aldehyde, and the betaine aldehyde is considered below to be converted into the basis of an intervention of the betaine aldehyde dehydrogenase by glycine betaine.
[0004] In recent years, the research which is going to obtain the vegetation which reinforced the resistance over environmental stress by genetic manipulation is active. for example, H.J.Bohnert ** -- Science Vol. -- 259, No.22, and p508-510 (1993) The mannitol synthetic enzyme of Escherichia coli was introduced into tobacco, and it has reported having given the salt atmosphere. Moreover, the betaine-aldehyde-dehydrogenase gene expression which is the gene which participates in composition of glycine betaine was raised to The Plant J.Vol.6 and p749-758 (1994), and although the example which was going to strengthen the vegetable salt atmosphere was indicated, since the gene of choline dehydrogenase was not introduced in fact, it did not lead to salt atmosphere improvement.
[0005] Although it is known that glycine betaine is participating in the resistance of vegetable environmental stress like the above, if what kind of gene is introduced, it is not known about the ability of environmental stress resistance to be given to this vegetation by increasing the glycine betaine in vegetation by being able to make the glycine betaine in vegetation increase, or introducing a foreignness gene artificially.
[0006]
[Problem(s) to be Solved by the Invention] Then, this invention tends to offer the vegetation which reinforced environmental stress resistance by the modifying-gene method, and the creation approach of the vegetation.
[0007]
[Means for Solving the Problem] In order that this invention person may solve the above-mentioned technical problem, variously The result of examination, By introducing into vegetation both choline

dehydrogenase gene which participates in composition of the glycine betaine which is one sort of the adaptation solute of the low-molecular organic compound nature considered to participate in the osmoregulation of a plant cell, and betaine-aldehyde-dehydrogenase gene, composition of glycine betaine, Or the composition could be reinforced and a header and this invention were completed for the ability of the resistance over environmental stress to be given to vegetation by that cause.
[0008] Therefore, this invention offers the vegetation by which the resistance over environmental stress was reinforced by introducing the choline dehydrogenase gene and betaine-aldehyde-dehydrogenase gene which participate in composition of glycine betaine. This invention is the creation approach of the above-mentioned vegetation again, and the approach characterized by introducing the choline dehydrogenase gene and betaine-aldehyde-dehydrogenase gene which participate in composition of glycine betaine at vegetation is offered.
[0009]
[Specific Explanation] Although especially the origin living thing of the choline dehydrogenase gene used in this invention and a betaine-aldehyde-dehydrogenase gene cannot be limited, for example, it can obtain from the high vegetation and high microorganism of environmental stress resistance on a target by birth, the gene of a microorganism, especially the bacteria origin is desirable. Cloning especially of the gene of the Escherichia coli origin has already been carried out (Lamark et al., Mol.Microbiol.Vol.5, p1049-1064, 1991), and it explains this invention concretely using the gene of the Escherichia coli origin in this invention as an example.
[0010] However, since what is necessary is just discovered by vegetable intracellular one, the gene of this invention is not limited to the gene of the Escherichia coli origin. The gene of the Escherichia coli origin follows the publication of the above-mentioned reference, and is 9kbp. Being able to obtain as a BamHI fragment, this fragment contains the choline dehydrogenase gene (betA) and the betaine-aldehyde-dehydrogenase gene (betB).
[0011] In this invention, although one sort of the freshwater blue-green-alga SHINEKOKOKKASU group (Synechococcus) which is the lower plants which has photosynthetic potential as vegetation is used, glycine betaine exists ranging from the cyanobacterium to a higher plant which is the lower plants, and since it is thought that it is participating in the resistance over vegetable environmental stress, it can apply this invention to all photosynthesis vegetation widely. As such vegetation, it is possible herb vegetation, such as tobacco, corn, a rice, wheat, barley, a tomato, a potato, an soybean, and cotton, and to apply to arboreous plants, such as a eucalyptus and an acacia, etc. widely further, for example.
[0012] the approach of the daily use for introducing a foreign gene into a plant cell, in order to introduce the aforementioned gene into the aforementioned vegetation, for example, the Agrobacterium method, and party Kurgan -- what is necessary is just to use approaches, such as law Moreover, also in order to reproduce for vegetation the plant cell into which the foreign gene was introduced, the approach by tissue culture in ordinary use can be used. In the example of this invention, the target gene was inserted in the shuttle vector which can be reproduced in both Escherichia coli and a blue-green-alga cell, and the approach of introducing this vector into a blue-green-alga cell was used.
[0013] Many vegetation has the biosynthesis ability of glycine betaine on the target by birth, according to this invention, it can reinforce the productivity of glycine betaine further in those vegetation, and can reinforce the resistance over environmental stress. It cannot be overemphasized that this invention is applicable to the vegetation which does not have the biosynthesis ability of glycine betaine substantially. The effectiveness that incorporation of a choline increases is also acquired by many vegetation's having the synthetic ability of the choline used as the middle raw material in the biosynthesis of glycine betaine, and introducing into a plant cell further the gene used by this invention, although composition of glycine betaine can be reinforced from a choline by introducing said gene used for such vegetation by this invention.
[0014] Glycine betaine exists in a cell etc., and since it is thought that it contributes to stabilization of an enzyme or the structural protein of a cell, the vegetation of this invention has resistance also to various environmental stress, such as not only the osmotic-pressure stress by a salt etc. but desiccation stress, low-temperature stress, elevated-temperature stress, etc.

[0015]
[Example] Next, an example explains this invention still more concretely.
Example 1 Cloning of the cloning gene of the SHINEKOKOKKASU sp. installation (1) gene of the gene to PCC7942 was performed according to Andresen et al., J.Gen.Microbiol.Vol.134, p1737-1764 (1988) and Lamark et al., Mol.Microbiol.Vol.165, and the approach indicated by p1059-1062 (1991). They are 9kbs by this approach. BamHI The DNA fragment was obtained from Escherichia coli (CSH26).
[0016] This DNA fragment contains the open reading frame (betA) which carries out the code of the choline dehydrogenase gene, the open reading frame (betB) which carries out the code of the betaine aldehyde dehydrogenase, and the open reading frame (betT) which carries out the code of the energy dependence transport system of a choline, as shown in drawing 1 .
[0017] (2) Production aforementioned 9kb of a manifestation shuttle vector BamHI Plasmid pBET was obtained by inserting a DNA fragment in the BamHI part of plasmid pUC303-Bm. The plasmid pUC303 is marketed as Escherichia coli / a SHINEKOKOKKASU shuttle vector, and pUC303-Bm is the plasmid which introduced 12bp(s) which contain a BamHI fragment in the EcoRI fragment of pUC303. To this BamHI part of plasmid pUC303-Bm, they are 9kbs in said plasmid pBET. The plasmid pCBET of 20kbs was obtained by inserting a BamHI part. In addition, the above-mentioned gene recombination actuation was performed, using Escherichia coli DH5alpha as a host.
[0018] (3) BG11 liquid medium which added Hepes-KOH (pH8.0) of 20mM(s) for SHINEKOKOKKASU (Synechococcus) sp.PCC7942 generally used by the genetic manipulation of the transformation blue-green alga of cyanobacterium (presentation:) [NaNO3] ; 1.5 g/l, and K2 HPO4 and 7H2 O;40 mg/l, and MgSO4 and 7H2 O;75 mg/l, CaCl2.2H2 O;36 mg/l, Ciuic Acid;6 mg/l, Ferric Ammonium Citrate;6mg, EDTA;1 mg/l, Na2 CO3 ; 20mg and MnSO4 - 7H2 O;2.5mg, ZnSO4 and 7H2 O; 222microg/l, CuSO4 and 5H2 O; 79microg/l, H3 BO4 ; 2.86 mg/l, NaMoO4 ; In 21microg/l, and Co (NO3)2 and 6H2 O; 500microg/l It cultivated under the continuous irradiation of the fluorescence white light at 30 degrees C. By mixing the shuttle plasmid pCBET with the cultivated blue-green-alga object, the transformation was carried out and it chose with streptomycin resistance. The blue-green alga increased under existence of 10microg [/ml] streptomycin was chosen. About the generated colony, it is 32P. - Said 9kbs which carried out the indicator It screened by Southern hybridization, using a DNA fragment (drawing 1) as a probe, and the electropositive clone was chosen.
[0019] next, in order to check the target gene expression, from the cultivated blue-green-alga cell H. All RNA is extracted by Aiba et al., J.Biol.Chem.Vol.256, and the approach indicated by p11905-11910 (1981). and Yang H. ** -- by the approach of a publication to Nucleic Acids Research vol.21 and p3337-3338 (1993) It is 32P as a probe. - RNA imprinted by Northern blot analysis using PstI and the BglII fragment (refer to drawing 1) which carried out the indicator was detected. In this trial, the electropositive clone as which the above was chosen is cultivated for two days by the culture medium which does not contain NaCl, and they are 200mM(s) to a pan. Detection was performed after cultivating by the BG11-choline culture medium containing NaCl.
[0020] As shown in the lane 2 of drawing 2 , the RNA transcript of about 9 kbs was detected about the blue-green alga of this invention, but about the cell in which the transformation was carried out by plasmid vector pUC303-Bm which did not insert a gene, as shown in the lane 1 of drawing 2 , a probe and RNA to hybridize were not imprinted. 9kbs inserted from the sizes of imprinted RNA being about 9 kbs That by which the DNA fragment was imprinted is presumed.
[0021] example 2 the cell of SHINEKOKOKKASU in which the transformation was carried out by vegetable incorporation vector plasmid pUC303-Bm or vegetable Plasmid pCBET of a property (1) choline by which the transformation was carried out -- 200mM or [containing NaCl] -- or you made it increase in BG11 culture medium which is not contained till the middle of logarithmic growth Choline transportation activity (incorporation of a choline) is a 10microM[14C]-choline (58.5mCi/mmol) by the approach indicated by Lamark et al., Mol.Microbiol.Vol.5, and p1049-1064 (1991) at 25 degrees C. It carried out by using. In addition, the endogenous choline was not detected in the frond increased under the nonexistence of a choline. This result is shown in drawing 3 .

[0022] As shown in A of drawing 3 , although the incorporation of a choline advanced under **-stress conditions, initial velocity and the intracellular choline content of 30 minutes after were high also in any of an object cell (cell in which the transformation was carried out by pUC303-Bm), and a bet gene content cell (cell in which the transformation was carried out by pCBET) about 40% in the pCBET support cell. This difference is a choline transport system [activity / in Escherichia coli] (5 Lamark T., Mol.Microbiol.Vol. p 1049 -1064 (1991)). It is the result of the betT gene in pCBET which is carrying out the code being functionally discovered. In all cases, incorporation of a choline was strongly checked by addition of 5microM of a carbonyl cyanide-p-trifluoro methoxypheny hydrazone (FCCP), and it was suggested that an energy dependence choline transport system exists in SHINEKOKOKKASU.
[0023] In addition, a hollow circle shows the result at the time of cultivating the cell by which the transformation was carried out by pUC303-Bm under the nonexistence of FCCP in drawing 3 . A hollow rectangular head shows the result at the time of cultivating the cell in which the transformation was carried out by pCBET under the nonexistence of FCCP. A black spot shows the result at the time of cultivating the cell by which the transformation was carried out by pUC303-Bm under existence of FCCP, and a result when a black rectangular head cultivates the cell in which the transformation was carried out by pCBET under existence of FCCP is shown.
[0024] As shown in B of drawing 3 , the choline was incorporated under salt stress conditions by only SHINEKOKOKKASU in which the transformation was carried out by pCBET. The energy dependence choline transport system which exists in the plasmlemma of SHINEKOKOKKASU changes with the existence of high concentration in a culture medium, and since composition of another side glycine betaine stabilized the film and enabled transportation of a choline under elevated-temperature concentration conditions, this is expected. In this case, ********** [because the feedback repression by the choline of the transport system in the inside of a bet gene content cell was canceled by use of the choline in glycine betaine composition].
[0025] (2) In SHINEKOKOKKASU sp.PCC7942 by which production trait conversion of the glycine betaine is not carried out, both the enzyme activity of choline dehydrogenase and the betaine aldehyde dehydrogenase was not detected, and glycine betaine was not accumulated at all under salt conditions. Since growth of SHINEKOKOKKASU sp.PCC7942 cell was prevented from 1mM by addition of a high-concentration choline under a non-stress condition and stress conditions, the choline of 100microM was added for production of the glycine betaine in the cell by which the transformation was carried out by pCBET.
[0026] The choline of this level did not affect growth of an object cell under various conditions. The cell of SHINEKOKOKKASU which carried out growth for two days in BG11 culture medium (BG11-choline culture medium) containing the choline of 100microM (till the middle of an exponential phase) was moved to the BG11-choline culture medium which has various NaCl concentration. It is 1N after the incubation for three days, and about a quaternary ammonium compound. H2 SO4 It extracted from the cell and was made to precipitate as the periodate (refer to Wall, JS et al., Anal.Chem.Vol.32, and p870-847 (1960)). The pellet was dissolved in t-butanol content D2 O of 600microL as an internal standard.
[0027] About analysis of a quaternary ammonium compound, it is 1H. It carried out using the JEOLJMN-500 fourier exchange NMR meter with the NMR spectral method. the volume of a cell -- BlumwaldE. ** -- it measured by Proc.Natl.Acad.Sci.USA Vol.80 and the approach indicated by p2599-2602 (1983). About the condensed cell, they are film impermeable paramagnetism elimination agent Na3 Fe (CN)6 and (20mM) Na2 Mn. It processed with the nitro KISAIDO spin probe THMPONE (2, 2, 6, and 6-tetramethyl piperidone-N-oxyl) penetrated to the freedom of 1mM under existence of EDTA (75mM), and the ESR signal of an intracellular spin probe was excited. The cell volume was measured also by the approach of Incharoensakidi A. et al., Plant Cell Physiol.Vol.29, and p1073-1075 (1988) again using 3H2 O and a [14C]-sorbitol.
[0028] A result is shown in drawing 4 . Setting to drawing 4 , A is the fourth class ammonium compound from an object (cell in which the transformation was carried out by pUC303-Bm). 1H An NMR spectrum is shown and beta shows it from the cell in which the transformation was carried out by

pCBET. Peaks b and c show a betaine and a choline, respectively. Only the cell the transformation was carried out [the cell] by pCBET so that clearly from drawing 4 compounded and accumulated glycine betaine.

[0029] The level of the glycine betaine in these cells changed with the salt concentration in a culture medium, and as shown in Table 1, it was crossed to 45mM(s) in about 3 mM(s) to NaCl concentration 375mM under non-stress conditions. The glycine betaine of such level gives sufficient protective effect for various cell functions (refer to Gerard H. et al., Plant Cell Physiol.Vol 29, p1073-1075 (1991) and Rhodes D. et al., Annu.Rev.Plant Physiol.Plant Mol.Biol.Vol.44, and p357-384 (1993)).

[0030]

[Table 1]

培地中NaCl濃度 (M)	ベタイン (mM)
0	3.2±0.4
0.1	4.5±0.6
0.2	8.8±0.7
0.3	24.1±2.0
0.375	45.1±1.9

[0031] (3) About the cell which carried out growth for two days (middle of a logarithmic growth phase) in the photosynthetic activity BG11-choline culture medium, it is 200mM. It moved to the BG11-choline culture medium which added NaCl, and culture was continued for four days. Since the photosynthetic activity of these cells was low, after moving to the fresh BG11-choline culture medium which does not contain NaCl for one day, PSI (photosystem I) and PSII (photosystem II) were measured in the Clark-type oxygen electrode in the generating list of the oxygen by photosynthesis.

[0032] The reaction medium contained PBQ (phenyl-1, 4-benzoquinone) of 1mM for measurement of MV (methyl viologen) of 400microM, or PSII electronic transportation activity for measurement of DCMU (3 -(3, 4-dichlorophenyl)- dimethyl urea) of 100microM, the sodium ascorbate of 1mM, DAD (2, 3, 5, 6-tetramethyl-beta-phenylenediamine) of 500microM, and PSI electronic transportation activity. Photosynthetic activity is 200mM(s) about a cell again. It measured also immediately after applying to the osmotic-pressure stress under existence of NaCl.

[0033] Although SHINEKOKOKKASU could be increased in the culture medium which contains NaCl by high concentration called 400mM(s), the cell was colored light yellow after increasing for four days under existence of NaCl of 300mM. This effectiveness was not observed in the cell in which the transformation was carried out by pCBET, but was still green. It is shown in drawing 6 by making this result into a black-and-white picture. In drawing 6 , right-hand side is the photograph of the culture of SHINEKOKOKKASU in which the transformation was carried out by pCBET, and left-hand side is the photograph of a contrast culture.

[0034] As expected, in the cell in which the transformation was carried out by pUC303-Bm compared with the cell in which the transformation was carried out by pCBET, the absorption spectrum showed the dramatic fall of a FIKOBI rhizome (Phycobilisome) [C-phycocyanin (lambda620-630nm)] and a chlorophyll content (data is not shown). O2 by photosynthesis any of the activity relevant to PSI and PSII to a generating list -- although -- in the cell in which the transformation was carried out by pCBET compared with the reference cell in which the transformation was carried out by pUC303-Bm, it was high. This result is shown in Table 2. In addition, the values of Table 2 were three averages of measured value, and the standard error was less than 5%.

[0035]

[Table 2]

反応	酸素の発生又は吸収（μmol O_2／mg Chl／h）	
	pUC303-Bm 形質転換細胞	pCBET形質転換細胞
Total ($H_2O \rightarrow CO_2$)	81(100)	98(121)*
PSI ($DADH_2 \rightarrow MV$)	145(100)	165(114)
PSII($H_2O \rightarrow PBQ$)	156(100)	217(139)

[0036] The following table 3 shows the photosynthetic activity acquired after moving the cell increased under non-stress conditions to high salt concentration conditions (200mMNaCl). Any measured value fell almost momentarily compared with the value from which it was obtained under salt stress nonexistence in the reference cell (especially, it of PSI). This fall was not remarkable in the cell in which the transformation was carried out by pCBET. In addition, the values of Table 3 were three averages of measured value, and the standard error was less than 5%.

[0037]

[Table 3]

反応	酸素の発生又は吸収（μmol O_2／mg Chl／h）			
	pUC303-Bm 形質転換細胞		pCBET形質転換細胞	
	-NaCl	+0.2M NaCl	-NaCl	+0.2M NaCl
Total ($H_2O \rightarrow CO_2$)	57(100)	40(68)*	52(100)	39(74)*
PSI ($DADH_2 \rightarrow MV$)	152(100)	96(63)	217(100)	177(82)
PSII($H_2O \rightarrow PBQ$)	71(100)	60(85)	78(100)	77(98)

[0038] According to the place which these observation results show, production of glycine betaine brings about a stabilization effect to a FIKOBI rhizome and PS complex under salt stress conditions.

[0039] (4) After proliferating the cell in which the transformation was carried out by the cell (contrast) by which the transformation was carried out by growth pUC303-Bm under salt stress conditions, and pCBET for two days in a BG11-choline culture medium, you made it increase in the BG11-choline culture medium of various NaCl concentration. All of the cell by which the transformation was carried out by the reference cell and pCBET are 0.3M. The proliferation rate almost same to NaCl was shown. However, in NaCl concentration higher than 0.3M, the cell by which the transformation was carried out by pCBET had the high proliferation rate compared with the reference cell (B and C of drawing 5). Among drawing 5 , in A, NaCl concentration 0.1M and B show 0.375M, and C shows the result of 0.4M. According to the place which a synthetic result shows, it was discovered in the SHINEKOKOKKASU cell, and functional protein generated the bet gene which exists in Plasmid pCBET. Furthermore, generally the glycine betaine produced by the cell produced advantageous effectiveness into a SHINEKOKOKKASU cell under salt stress conditions.

[Translation done.]

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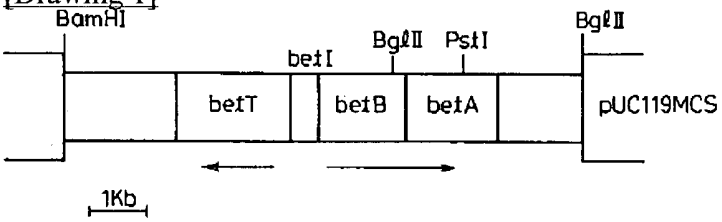
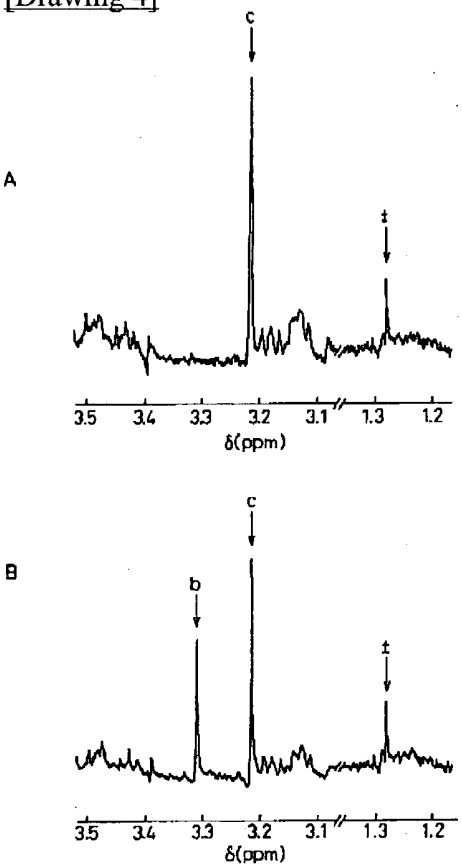
DRAWINGS

[Drawing 1]

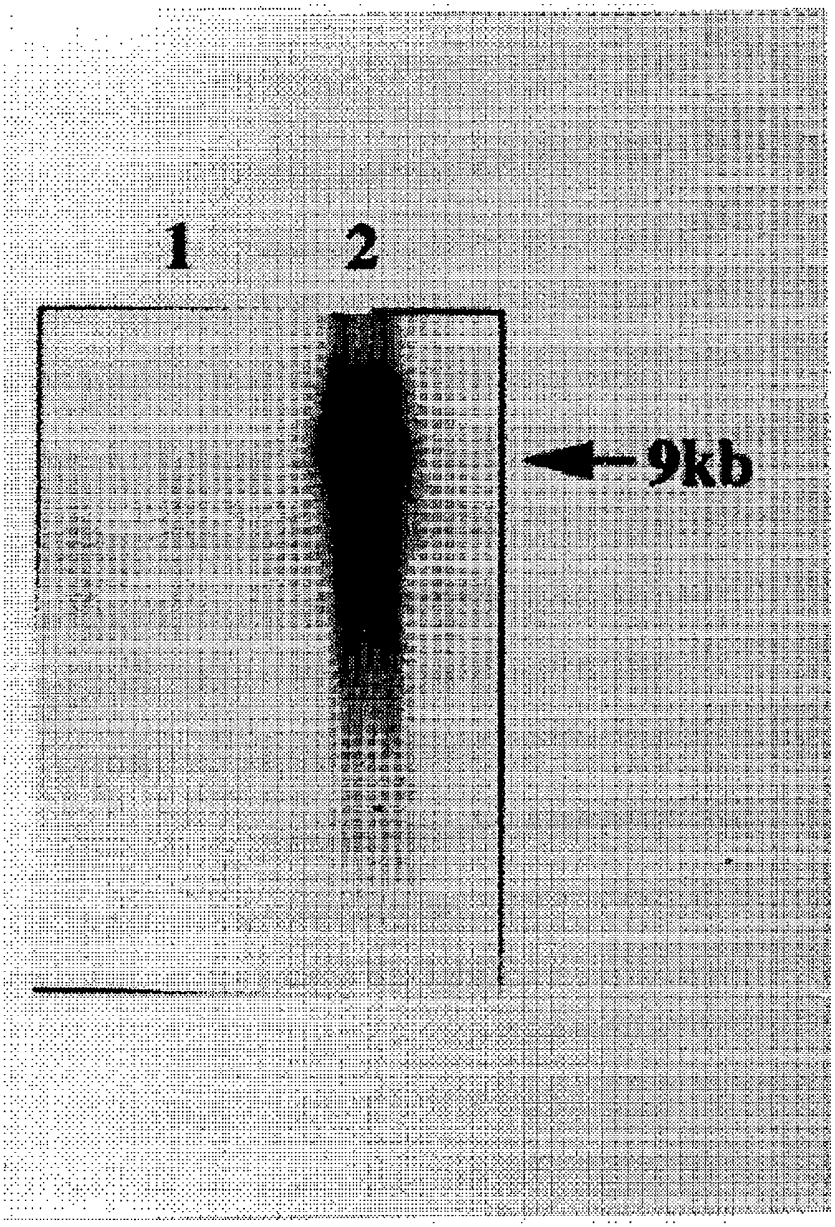


[Drawing 4]



[Drawing 2]

図面代用写真



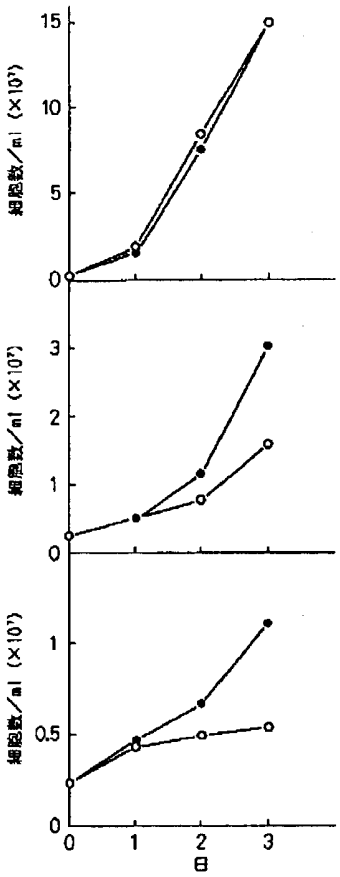
写　　真

[Drawing 5]

A

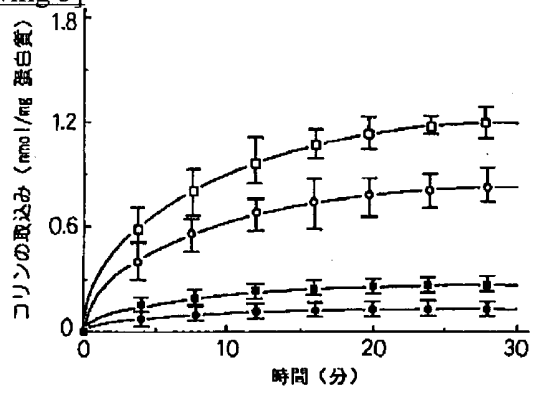
B

C



[Drawing 3]

A



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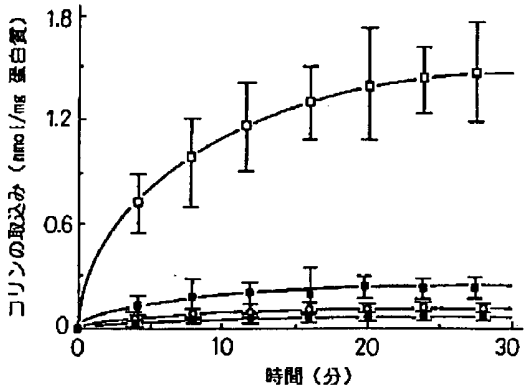

[Drawing 6]

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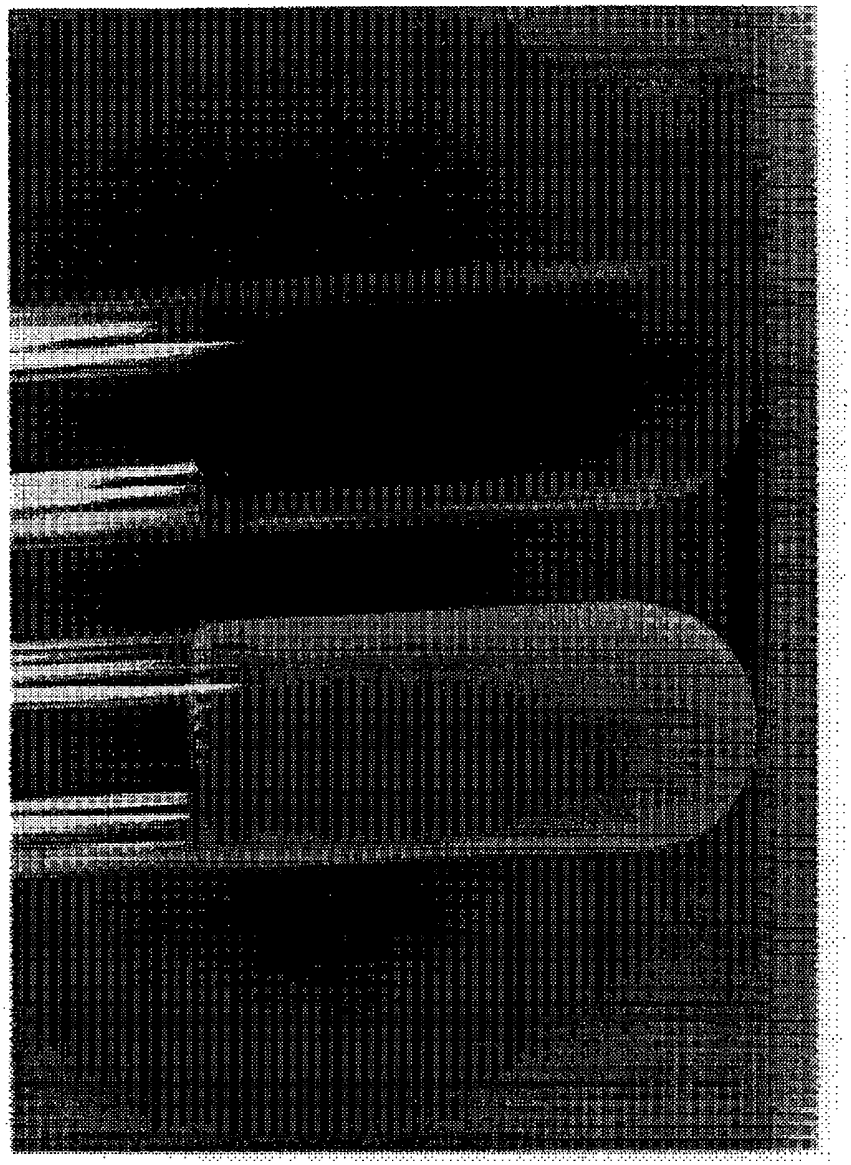

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CLAIMS

[Claim(s)]
[Claim 1] Vegetation by which the resistance over environmental stress is reinforced by introducing the choline dehydrogenase gene and betaine-aldehyde-dehydrogenase gene which participate in composition of glycine betaine.
[Claim 2] The approach characterized by introducing the choline dehydrogenase gene and betaine-aldehyde-dehydrogenase gene which are the creation approach of vegetation according to claim 1, and participate in composition of glycine betaine at vegetation.

[Translation done.]